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Synthesis of Several Light-Harvesting Complex I Polypeptides Is Blocked by Cycloheximide in Symbiotic Chloroplasts in the Sea Slug, *Elysia chlorotica* (Gould): A Case for Horizontal Gene Transfer Between Alga and Animal?

JEFFREY J. HANTEN[1,2] AND SIDNEY K. PIERCE[2,*]

[1] *Department of Biology, University of Maryland, College Park, Maryland 20742; and*
[2] *Department of Biology, University of South Florida, Tampa, Florida 33620*

Abstract. The chloroplast symbiosis between the ascoglossan (=Sacoglossa) sea slug *Elysia chlorotica* and plastids from the chromophytic alga *Vaucheria litorea* is the longest-lived relationship of its kind known, lasting up to 9 months. During this time, the plastids continue to photosynthesize in the absence of the algal nucleus at rates sufficient to meet the nutritional needs of the slugs. We have previously demonstrated that the synthesis of photosynthetic proteins occurs while the plastids reside within the diverticular cells of the slug. Here, we have identified several of these synthesized proteins as belonging to the nuclear-encoded family of polypeptides known as light-harvesting complex I (LHCI). The synthesis of LHCI is blocked by the cytosolic ribosomal inhibitor cycloheximide and proceeds in the presence of chloramphenicol, a plastid ribosome inhibitor, indicating that the gene encoding LHCI resides in the nuclear DNA of the slug. These results suggest that a horizontal transfer of the LHCI gene from the alga to the slug has taken place.

Introduction

Most alga-animal symbioses are extracellular associations between two genetically distinct organisms. The alga is usually located extracellularly or enclosed within vacuoles inside the animal's cells. Rarer, but not uncommon, are intracellular symbioses occurring with intact algal chloroplasts that are captured by specialized cells within the animal. In particular, several species of ascoglossan (=Sacoglossa) (Opistobranchia) sea slugs capture intact, functional plastids from their algal food source and retain them within specialized cells lining the mollusc's digestive diverticula. This phenomenon has been termed chloroplast symbiosis (Taylor, 1970) or kleptoplasty (Clark *et al.*, 1990). The sequestered plastids continue to photosynthesize for periods ranging from a few days to a few months, depending on the species (Greene, 1970; Hinde and Smith, 1974; Graves *et al.*, 1979; Clark *et al.*, 1990).

The longest such association, lasting as long as 9 months, is found in *Elysia chlorotica* (Gould), which obtains symbiotic plastids from the chromophytic alga *Vaucheria litorea* (C. Agardh) (West, 1979; Pierce *et al.*, 1996). The association begins at metamorphosis of the slug from planktonic veliger to juvenile. In laboratory cultures, filaments of *V. litorea* must be present for metamorphosis to take place (West *et al.*, 1984). Veligers home in, attach to the filaments, and metamorphose into juvenile slugs over the next 24 h. The juveniles eat the algal filaments and sequester the chloroplasts within one of at least two morphologically distinct types of epithelial cells lining the walls of the digestive diverticula (West *et al.*, 1984). Once the plastids are sequestered, the slugs can sustain photosynthesis at rates sufficient to satisfy the nutritional needs for the complete life cycle of the slug, when provided with direct light and carbon dioxide (Mujer *et al.*, 1996; Pierce *et al.*, 1996).

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* To whom correspondence should be addressed. E-mail: pierce@chumal.cas.usf.edu

Abbreviations: CAP, chloramphenicol; CHX, cycloheximide; FCPC, fucoxanthin chlorophyll *a/c* binding proteins; LHC, light-harvesting complex; PSI; photosystem I.

Even in nature the slugs obtain most of their energy from photosynthesis (West, 1979).

The longevity of this relationship in *E. chlorotica* makes it especially interesting. Photosynthesis requires the continuous synthesis of a variety of chloroplast proteins because many of them, including those used in light harvesting, are rapidly degraded and must be replaced (Greenberg *et al.*, 1989; Mattoo *et al.*, 1989; Barber and Andersson, 1992; Wollman *et al.*, 1999). Furthermore, photosynthesis requires the interaction of as many as 1000 proteins, only about 13% of which are coded in the plastid genome (Martin and Herrmann, 1998). In the plant cell, substantial nuclear input is required to sustain photosynthetic function, in the form of direct coding of the proteins as well as providing the means for their intracellular transport and regulation (Berry-Lowe and Schmidt, 1991; Wollman *et al.*, 1999). Considering the level of nuclear and extra-plastid input required, it is not surprising that the longevity of the plastids in most kleptoplastic slugs is relatively short. However, several photosynthetic proteins are synthesized in the sequestered plastids of *E. chlorotica* (Pierce *et al.*, 1996), including the large subunit of RuBisCO, D1, D2, CP43, cyt *f* and others (Pierce *et al.*, 1996; Mujer *et al.*, 1996; Green *et al.*, 2000). Although all of the synthesized plastid proteins identified to date are plastid encoded (Mujer *et al.*, 1996; Pierce *et al.*, 1996; Green *et al.*, 2000), two groups of synthesized plastid proteins can be distinguished pharmacologically: those inhibited by cycloheximide (CHX), an 80S cytosolic ribosome inhibitor (Obrig *et al.*, 1971), and those inhibited by chloramphenicol (CAP), which inhibits protein synthesis on 70S plastid and mitochondrial ribosomes (Lamb *et al.*, 1968; Stone and Wilke, 1975).

Because the inhibition by CHX suggests that the genes for several plastid proteins must reside in the nuclear DNA, we have done some experiments to identify these proteins and test that possibility. Our present study reports the identification of several of the CHX-blocked proteins as members of the light-harvesting complex 1 (LHC1), a family of pigment-binding proteins responsible for collecting radiation energy from sunlight and transferring it to photosystem 1 (PS1). LHC1 proteins are encoded by the *Lhca* genes in the nuclear genome of all the plants and algae whose genomes have been examined (Jansson, 1994, 1999; Green and Durnford, 1996; Durnford *et al.*, 1999; Wollman *et al.*, 1999). This result suggests that the LHC1 genes have been somehow transferred from the algal nucleus to the slug's DNA.

Materials and Methods

Animals and alga

Specimens of *Elysia chlorotica* were collected in both the spring and fall from an intertidal marsh near Menemsha Pond on the island of Martha's Vineyard, Massachusetts. The slugs were maintained in 10-gallon aquaria at 10 °C in aerated, artificial seawater (ASW; Instant Ocean, 925-1000 mosm) on a 16/8-h light/dark cycle (GE cool-white fluorescent tubes, 15 W).

Sterile cultures of *Vaucheria litorea* were maintained in enriched ASW (400 mosm) [modified from the F/2 medium (Bidwell and Spotte, 1985)]. The alga was grown at 20 °C on a 16/8-h light/dark cycle (GE cool-white fluorescent tubes; 40 W), and the medium was changed weekly.

Inhibitor treatments and plastid protein labeling

All reagents used were molecular bio-grade (DNase-, RNase-, and protease-free) purchased from Sigma unless otherwise noted. Effective concentrations of CHX and CAP were determined empirically with initial dose-response curves (Pierce *et al.*, 1996). CHX (2 mg ml^{-1}) was used to inhibit protein synthesis on 80S cytosolic ribosomes; CAP (160 μg ml^{-1}; stock concentration 50 mg ml^{-1} in absolute ethanol) was used to inhibit translation on 70S plastid and mitochondrial ribosomes. Two to four slugs, total wet weight about 1.25 g, were placed into glass scintillation vials containing ASW (1000 mosm) and the appropriate inhibitor, and incubated under intense light (150 W, GE Cool Beam incandescent indoor flood lamp) at 20 °C in a gently agitating water bath. After 1 h, 20 μCi ml^{-1} [^{35}S]-methionine (0.7 MBq ml^{-1}, trans-[^{35}S]-methionine, ICN) was added, and the slugs were incubated for an additional 6 h, previously demonstrated to provide ample time to incorporate radioactive label into the plastid proteins (Pierce *et al.*, 1996). Additional slugs were incubated in 0.025% ethanol/ASW (v/v) solution plus [^{35}S]-methionine to serve as a control for the carrier in CAP treatments.

Chloroplast isolation and protein separation

Chloroplasts were isolated from slugs by using a centrifugation protocol. The slugs were homogenized in the presence of the mucolytic agent N-acetyl-cysteine (500 m*M*), and the homogenate was filtered successively through cheesecloth, Miracloth (Calbiochem), and then nylon mesh (60 μm to 10 μm) to remove large debris and the copious amount of mucus the animals produce. The plastids were purified on a pre-formed, 25% Percoll (v/v) gradient, which provides a very pure fraction containing large numbers of intact plastids (Pierce *et al.*, 1996). In this experiment, the lowest green band containing labeled plastids was isolated from the gradient by using a flamed Pasteur pipette, and residual Percoll was removed by centrifugation. The purified chloroplast pellets were resuspended, lysed by freeze-thawing, and stored at −20°C until use. The incorporation of radioactive label was determined by a liquid scintillation counter (Beckman LS6000IC), and the protein content was determined using the modified Lowry assay (Peterson, 1977). The resulting specific activity was calculated as counts per minute (cpm) (μg protein)$^{-1}$. Chlorophyll con-

tent was determined by extracting the pigment in 80% acetone, then measuring the extract absorbance spectrophotometrically at 652 nm. The results were calculated as micrograms per microliter according to standard equations (Joyard *et al.*, 1987).

Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) autoradiography was used to assess the effects of CHX and CAP on the pattern of protein synthesis. The plastid lysates obtained from the above procedure were boiled for 2 min in Tris-HCl (pH 6.8)–10% SDS (w/v) buffer containing 5% β-mercaptoethanol (β-ME) (v/v). The solubilized proteins were loaded in equal amounts onto 15% SDS-polyacrylamide gels and separated by electrophoresis (Laemlli, 1970). The gels were stained with Coomassie brilliant blue, dried, and exposed to film (Kodak Biomax MR) for 2 to 30 days at −80°C, depending on the level of radioactive label present. Approximate molecular masses of the proteins were determined by comparison to the migration distances of known molecular weight standards (BioRad, broad-range kaleidoscope) run in adjacent lanes on each gel.

Immunoblot identification of plastid proteins

After the plastid isolation and protein separation *via* SDS-PAGE as described above, the proteins were electrophoretically transferred (30 V, 4 °C, overnight) to PVDF membranes (Immobilon-P; Millipore) (Towbin *et al.*, 1979). As additional controls, *V. litorea* chloroplasts [isolated and purified using a 30% to 75% Percoll step gradient as previously described (Pierce *et al.*, 1996)] and thylakoids from the red alga *Porphyridium cruentum* (generously donated by Professor Elisabeth Gantt, University of Maryland), were lysed, and the proteins were separated electrophoretically and transferred to membranes as above. The membranes were blocked with 5% (w/v) dehydrated milk dissolved in Tris-buffered saline (TBS) (Tris-base 50 m*M*, NaCl 0.9%, pH 7.5) for 1 h at room temperature, washed twice in TBS for 10 min, and treated with primary antibody for 1 h. In this case, the primary antibody was a polyclonal antibody to LHCI which was produced in a rabbit using a 22-kDa, recombinant LHCI polypeptide produced from a clone of the *LhcaRI* gene of *P. cruentum* (Grabowski *et al.*, 2000) (also provided by Professor Gantt) ["*RI*" indicating it is a rhodophyte gene (Tan *et al.*, 1997a)] as the antigen combined with Freund's adjuvant in a standard immunization procedure. After binding of the primary antibody, the membranes were washed twice as above and incubated with secondary antibody, anti-rabbit conjugated hydrogen peroxidase, for 1 h. After washing, the bands were visualized with a 4-chloro-1-napthol and hydrogen peroxide reaction according to manufacturer's instructions. The immunolabeled western blots were exposed to film as described above to identify the coincidence of antibody binding and radioactive incorporation in the presence of each inhibitor.

As a control to confirm that the CAP was blocking plastid-directed protein synthesis and that CHX was not, parallel measurements were run to monitor cytochrome *f* (cyt *f*) synthesis. Earlier experiments conducted on *E. chlorotica* have demonstrated that cyt *f* is synthesized in the slugs and is encoded in the plastid DNA (Green *et al.*, 2000). Thus, if CHX and CAP are working as expected, their effect on cyt *f* and any nuclear-encoded proteins should be opposite. Anti-cyt *f*, raised to *P. cruentum* cyt *f*, was also a gift of Professor Gantt.

Immunoprecipitations

Immunoprecipitations were conducted to confirm the identity of the radioactive immunolabeled bands on the western blots, using a modified version of the protocol previously used to precipitate proteins from isolated *E. chlorotica* plastids (Pierce *et al.*, 1996). Plastid proteins were solubilized in lysing buffer (10 m*M* Tris-HCl, 10 m*M* EDTA, 150 m*M* NaCl, 1 m*M* PMSF, 1% (v/v) Nonidet P-40, pH 8.0), using equal amounts of chlorophyll per sample, mixed with a small amount of Protein-A Sepharose beads to eliminate nonspecific binding, and incubated on ice with occasional agitation. The beads were removed by centrifugation and discarded, the supernatant was saved, and the appropriate antibody was added to the lysate and rotated overnight (4 °C). Protein-A beads, swelled in washing buffer (50 m*M* Tris-HCl, 5 m*M* EDTA, 150 m*M* NaCl, 1 m*M* PMSF, 0.1% (v/v) Nonidet P-40, pH 8.0), were added the following morning and rotated (3 h, room temperature). The antigen–antibody–protein-A Sepharose bead complexes were washed several times in washing buffer and removed by centrifugation. In the case of cyt *f*, the antigen–antibody–protein-A Sepharose bead complexes were resuspended in 10.0 *M* urea, 10% SDS (w/v), 5% β-ME (v/v), pH 12.5, and boiled for 10 min to liberate the cyt *f* antigen. The solution was centrifuged, the supernatant was removed, and the beads were discarded. The supernatant proteins were separated by SDS-PAGE as described above, and the gel was autoradiographed.

The LHCI antibody-antigen complex could not be broken efficiently with any treatment, which prevented the visualization of the labeled LHCI proteins *via* SDS-PAGE. Although this was unexpected, it is not unusual and may have been caused by a number of factors. The presence of several different LHCI polypeptides with varying isoelectric points, ranging between 4.5 and 9.5 (De Martino *et al.*, 2000), makes it very difficult to create optimal reaction conditions for each one. The polyclonal antibody molecules bind to all the LHCI polypeptides as well as to each other, creating a large antigen-antibody complex with a core inaccessible to the chemicals necessary to liberate the antigen. Very few

researchers have attempted LHC immunoprecipitations because of the pitfalls involved in precipitating inner-membrane proteins (Anderson and Blobel, 1983). Instead, other protocols have been designed using mild detergents to extract intact photosystem holocomplexes from the thylakoids, followed by protein separation on sucrose density gradients (Fawley and Grossman, 1986; Buchel and Wilhelm, 1993; Wolfe *et al.*, 1994; Schmid *et al.*, 1997). These isolations require large amounts of starting material (Schmid *et al.*, 1997) that greatly exceed what is available to us in the slugs. So, instead, we used the LHCI antibody to demonstrate that LHCI had incorporated radioactivity.

Following the procedure described above, the protein A Sepharose beads were reacted with anti-LHCI and then with a radiolabeled plastid protein extract. The antigen–antibody–protein-A Sepharose bead complexes were repeatedly washed by centrifugation until the radioactivity in the supernatant was reduced to background. The washed antigen–antibody–protein-A Sepharose bead complexes were resuspended in optifluor (Packard), and radioactivity was determined by a scintillation counter. Controls for nonspecific binding to protein-A Sepharose beads were conducted with the same procedure, but without the addition of the LHCI antibody. Counts per minute resulting from nonspecific binding were subtracted from experimental values for each inhibitor treatment and controls, and the final data were converted to cpm $(\mu g \text{ chlorophyll})^{-1}$ $(\mu g \text{ protein})^{-1}$. The normalized data were averaged and expressed in terms of percent of control for each inhibitor.

Results

Plastid protein synthesis and identification

The Coomassie-stained SDS-PAGE gels of protein extracts from isolated slug plastids were similar to controls regardless of the inhibitor present, either CHX or CAP, indicating no difference in the protein composition of the plastids after treatment (Fig. 1). However, autoradiograms of SDS-PAGE gels of plastid proteins extracted from slugs incubated in the presence of [^{35}S]-methionine indicate that very different patterns of protein synthesis occur in the slugs between controls and inhibitors as well as between inhibitors (Fig. 2). CHX has a profound effect on protein synthesis, preventing synthesis of the majority of the protein bands labeled in the absence of inhibitor (Fig. 2, CON), whereas the synthesis of many more labeled bands occurs in the presence of CAP. Furthermore, these protein bands differ from those visualized in the CHX treatments (Fig. 2).

Verification of inhibitor effects

Cyt *f* antibodies reacted with a protein band synthesized in the presence of CHX on western blots at approximately 36 kDa (Fig. 3). Immunoprecipitations using anti-cyt *f*

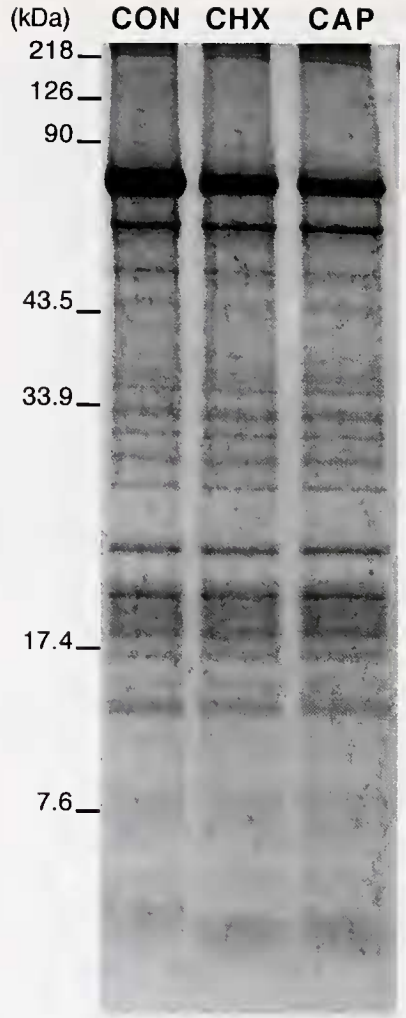

Figure 1. Coomassie brilliant blue-stained 15% SDS-PAGE gel of proteins extracted from isolated *Elysia* chloroplasts. The protein bands visualized are identical regardless of the inhibitor treatment, CHX or CAP (CON refers to control). Approximate molecular weights are indicated to the left.

identify a band with a molecular weight corresponding to cyt *f*, confirming its identity (Fig. 4). Autoradiograms of the same gels show [^{35}S]-methionine incorporation into cyt *f* in the presence of CHX, but not in the presence of CAP (Fig. 5).

The anti-LHCI we made to *Porphyridium cruentum* recombinant LHCI recognized both the recombinant LHCI antigen (Fig. 5A, lane 1) and the LHCI polypeptides from *P. cruentum* thylakoids (Fig. 5B, lane 2). Six polypeptide bands were identified in *P. cruentum*, ranging in approximate molecular weights from 19 to 24 kDa (Fig. 5B, lane 2), sizes consistent with those previously described for the LHCI polypeptides in this species (Tan *et al.*, 1995). The antibody bound onto western blots of plastid proteins from *Vaucheria litorea* and *Elysia chlorotica*, with or without the CHX and CAP treatments (Fig. 5C, lanes *V. lit.*, CON,

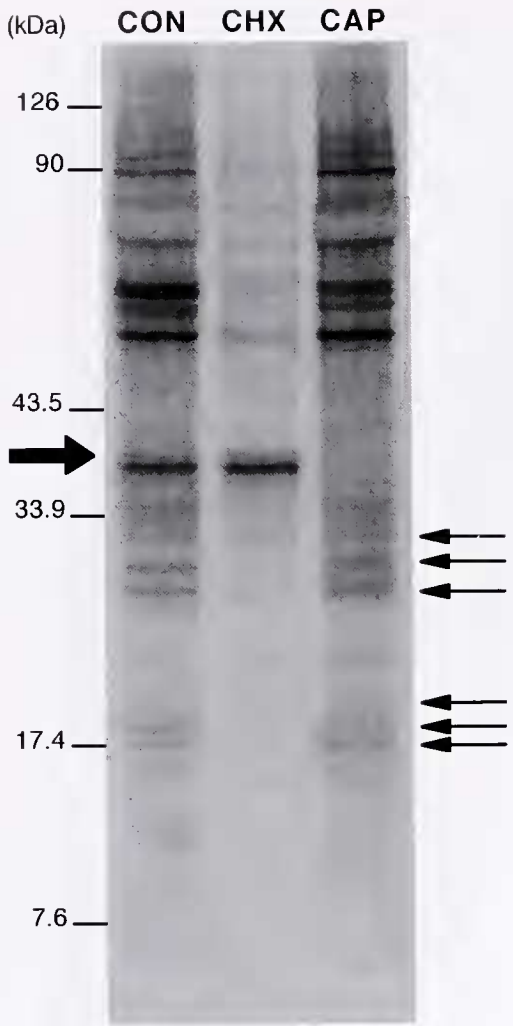

Figure 2. Autoradiograph of plastid proteins separated by SDS-PAGE gel run under the same conditions as those depicted in Figure 2. The plastid proteins incorporating [^{35}S]-methionine label differ following treatment with CHX or CAP. The control (CON) represents chloroplast proteins isolated from slugs without inhibitor treatment. Arrows identify the approximate positions of cyt *f* (large arrow) and the LHCI (small arrows) proteins.

CHX, CAP). As expected, the six polypeptide bands bound by the anti-LHCI in *V. litorea* and *E. chlorotica* plastids have a slightly greater size range—18 to 32 kDa—than those identified in *P. cruentum*. These same antibody-labeled bands from *E. chlorotica* plastid proteins incorporate radioactive label in the presence of CAP, but incorporation is blocked by the presence of CHX (Fig. 6).

The amount of radiolabel precipitated by anti-LHCI from the slug plastid extracts following CHX treatment is only 2% of the control level, indicating a reduction in LHCI synthesis (Fig. 7). In contrast, the LHCI proteins in CAP-treated slugs incorporated [^{35}S]-methionine at 92% of control rates, more than 40-fold higher than the level found in CHX treated animals (Fig. 7).

Discussion

LHCI, a family of plastid polypeptides essential for photosynthesis, is synthesized while *Vaucheria litorea* chloroplasts reside within the cells of the digestive diverticula of *Elysia chlorotica*. In addition, our data indicate the LHCI polypeptides are probably the products of genes located in the host-cell nuclear genome because their synthesis is inhibited by the cytosolic ribosome inhibitor, CHX, but not by the presence of the plastid ribosome inhibitor, CAP. This remarkable result would not be surprising in a plant or algal species since the LHCI polypeptide family's genes, *Lhca1-Lhca6*, reside in the nuclear DNA of all plants and algae examined to date (Jansson, 1994; Green and Durnford,

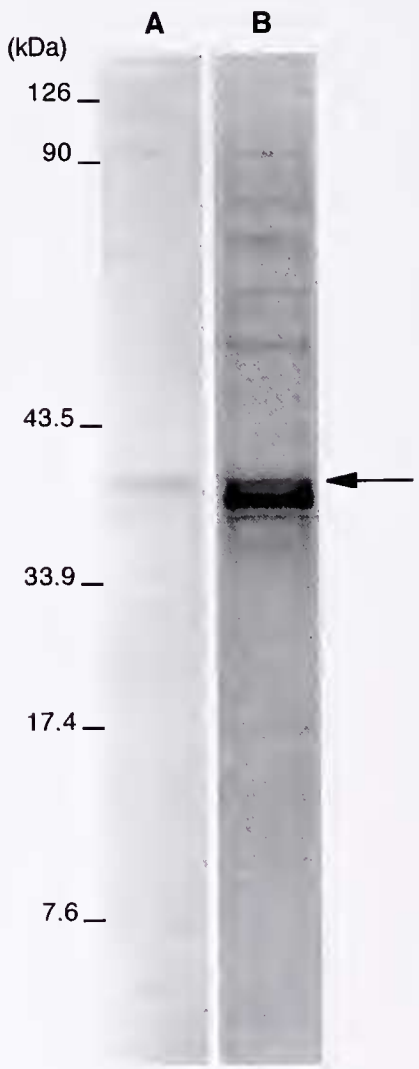

Figure 3. Immunoblot labeled with antibody to cyt *f* (A), and its corresponding autoradiograph (B). The slugs were exposed to CHX and the proteins were labeled as described in the methods. Anti-cyt *f* binds at approximately 36 kDa, coincident with a radiolabeled protein. The arrow indicates the autoradiograph band corresponding to the position of cyt *f*.

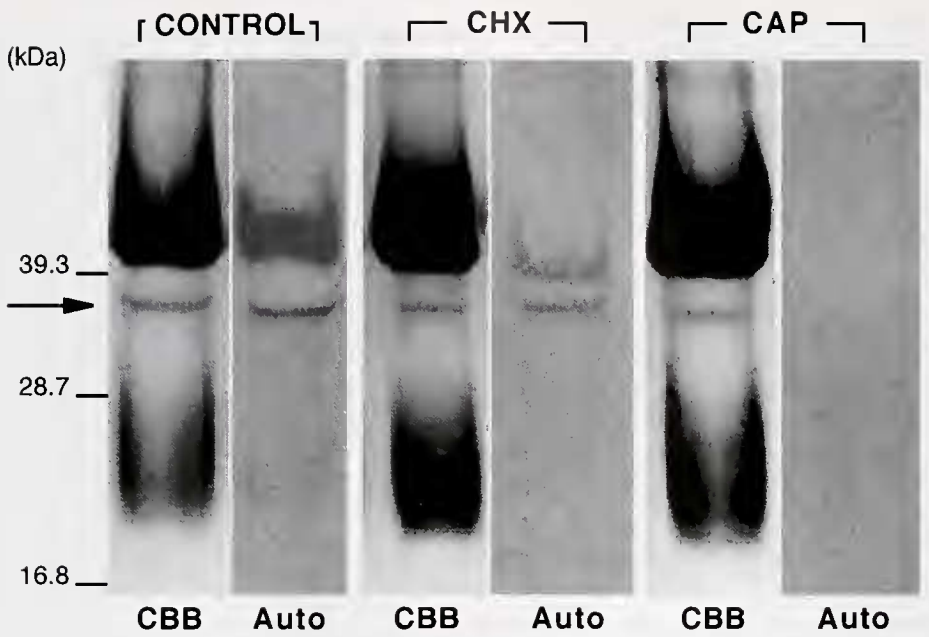

Figure 4. Immunoprecipitation of cyt *f*. Coomassie brilliant blue (CBB)-stained gels of proteins precipitated with anti-cyt *f* from chloroplast extracts from slugs subjected to no inhibitor (Control), to CHX, or to CAP, and their corresponding autoradiographs (Auto). The arrow indicates the position of cyt *f*. Large bands above and below cyt *f* are the heavy and light chains of the antibody, respectively. The radioactivity corresponding to the antibody bands in control and CHX is probably undissociated cyt *f*.

1996; Durnford *et al.*, 1999; Jansson, 1999; Wollman *et al.*, 1999). However, the synthesis of LHCI directed by an animal's genome indicates that genes have been transferred into the slug DNA.

Although surprising, the site of synthesis and the identification of LHCI seem to be without question as long as inhibitor and antibody specificity are not problems. Both CHX and CAP have been used in a wide array of studies, and their sites of action are well established. In fact, they have been used, exactly as we have done here, to establish that the site of synthesis of the "light harvesting chlorophyll protein" (=LHCI) occurs on 80s cytoplasmic ribosomes in *Phaeodactylum tricornutum* (Fawley and Grossman, 1986).

There are several reasons to conclude that our antibody is specific. We raised the antibody against the red alga LHCI not only because it was available, but also because the chromophytes, the taxonomic group of *V. litorea*, probably arose through a secondary symbiosis from a red alga (Rieth, 1995; Green and Durnford, 1996; Palmer and Delwiche, 1996; Martin and Herrmann, 1998; Delwiche, 1999). Furthermore, *Porphyridium cruentum* LHCI possesses both sequence homologies and immunological relatedness to the chromophytic light-harvesting proteins (Wolfe *et al.*, 1994; Rieth, 1995; Tan *et al.*, 1997b). Thus, a polyclonal antibody raised to a rhodophyte LHCI should have a good chance of specifically recognizing the LHCI polypeptides in *V. litorea*. Our results indicate that the anti-LHCI binds the *P. cruentum* recombinant LHCI, the antigenic source for the antibody, as well as all six of the native *P. cruentum* LHCI proteins (Tan *et al.*, 1995; Grabowski *et al.*, 2000) in control immunoblots of extracted thylakoids. The anti-LHCI immunoblots of *E. chlorotica* and *V. litorea* also identified six protein bands with a greater size range than the LHCI proteins identified in *P. cruentum*. Those bands are consistent with the sizes of LHCI polypeptides from many species (Gantt, 1996; Jansson, 1999; Wollman *et al.*, 1999), and no other bands were labeled by the antibody. Seeing six LHCI proteins is not surprising, because LHCI is typically found in multiple homologs in algae, ranging from two in one species of Xanthophyceae (Buchel and Wilhelm, 1993) to at least six paralogs in some rhodophytes (Tan *et al.*, 1995), and as many as eight in the chromophyte *Heterosigma carterae* (Durnford and Green, 1994). With few exceptions [such as in *Euglena gracilis* (Jansson, 1994)], each is encoded by a separate, nuclear gene belonging to the *Lhc* super-gene family (Jansson, 1999). Thus, location of the gene aside, the presence of six LHCI proteins in the endosymbiotic plastids in *E. chlorotica* is not surprising.

It seems clear that each of the bands immunodecorated by anti-LHCI corresponds to a single LHCI polypeptide and not a dimer. LHCI dimers can result from their association with other LHC proteins and their respective photosystems *in situ*, and they do not always readily dissociate under the denaturing conditions of SDS-PAGE (Tan *et al.*, 1995). If LHCI dimers were present here, they should have minimum molecular weights of about 36 kDa, corresponding to dou-

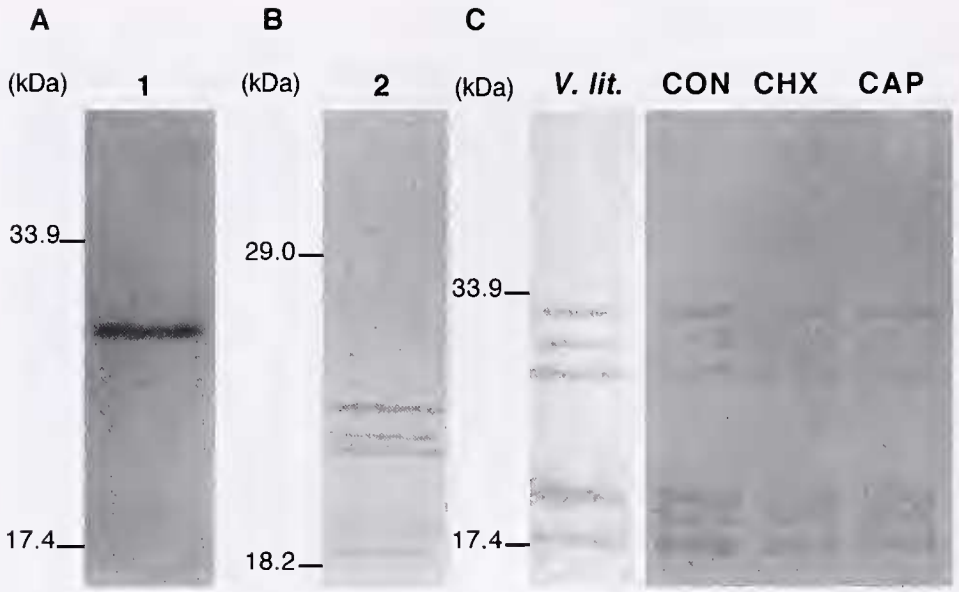

Figure 5. Immunoblots testing the antibody raised to *Porphyridium cruentum* LHCI. (A) Anti-LHCI binds the recombinant 22 kDa *Lhca* R1 product from *P. cruentum* (lane 1). Its appearance as a 28–30 kDa protein in SDS-PAGE and subsequent immunoblots results from the addition of a 33 amino acid N-terminal fusion in the recombinant protein (Grabowski *et al.*, 2000). (B) Anti-LHCI binds LHCI polypeptides extracted from *P. cruentum* thylakoids (lane 2). (C) *Vaucheria litorea* (lane *V. lit.*) and *Elysia chlorotica* plastid proteins have six bands binding the anti-LHCI identical in size to each other. All six proteins are present in the slugs regardless of the inhibitor treatment [lanes CON (control), CHX and CAP]. Molecular weights are indicated to the left of (A), (B), and (C).

ble the molecular weight of the smallest immunolabeled band. However, the largest of the six immunolabeled bands present in the gels is about 32 kDa, seemingly too small to be an LHCI dimer.

Other dimers might form with a number of photosystem I (PSI) proteins due to the close association of LHCI with the PSI subunits that compose the PSI-LHCI holocomplex (Wollman *et al.*, 1999; Jansson, 1999). This also does not seem to be the case here. Anti-PSI, raised against the cyanobacteria PSI holocomplex (again, courtesy of Professor Gantt), binds a single 10-kDa protein band on western blots of *E. chlorotica* plastid proteins (data not shown). The combination of this PSI polypeptide with any of the three smaller bands (18–20 kDa) that react with the anti-LHCI could form a dimer with molecular weights comparable to each of the three larger polypeptides (28–32 kDa). However, since anti-PSI and anti-LHCI do not co-label any bands, an LHCI-PSI dimer is unlikely.

An additional possibility might be that one of the bands could be another LHC-type protein possessing immunological similarities to LHCI, such as the fucoxanthin chlorophyll *a/c* binding proteins (FCPC) found in chromophytes or light-harvesting complex II (LHCII) proteins. In fact, our previous work has demonstrated the presence of FCPC in plastids of both *E. chlorotica* and *V. litorea*. However, the size of the FCPC protein identified there does not correspond to the weights of the proteins bound by the anti-LHCI used here (Pierce *et al.*, 1996; Green *et al.*, 2000). Furthermore, previous attempts to demonstrate FCPC synthesis with radioactive labels in the slugs have not yielded positive results (Pierce *et al.*, 1996).

The LHCII family of polypeptides is closely related to LHCI, performing similar functions in photosystem II to those performed by LHCI in PSI. The LHC II genes are in the same nuclear-encoded *Lhc* super-gene family (Jansson, 1999) and share sequence homologies with those genes encoding LHCI (Durnford *et al.*, 1999; Jansson, 1999; Wollman *et al.*, 1999). There is, however, a clear separation in the phylogenies of LHCI and LHCII (Durnford *et al.*, 1999), indicating some degree of dissimilarity between the two proteins. Nevertheless, the possibility seems to remain that the proteins bound by our antibody could be from LHCII.

Of the LHCII components, CP24, CP26, and CP29 contain the most sequence similarities to the LHCIs (Green and Durnford, 1996) and have molecular weights, 25–30 kDa (Wollman *et al.*, 1999), that roughly correspond to those of the three largest polypeptides identified in our anti-LHCI immunoblots of *E. chlorotica* and *V. litorea* plastid proteins (28–32 kDa), which appear to be slightly larger than most LHC proteins in chromophytes (Green and Durnford, 1996). An LHCII antibody derived from pea (generously donated by Dr. Kenneth Cline, University of Florida) was unreactive in our immunoblotting protocol (data not shown). This

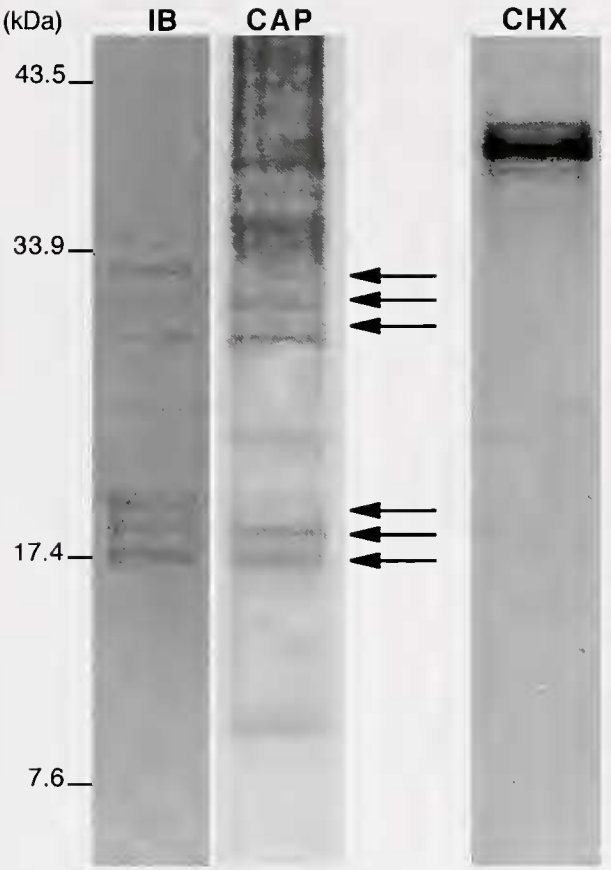

Figure 6. Immunoblot (IB) of LHCI synthesized in the presence of CAP and ^{35}[S]-methionine, and its corresponding autoradiograph (CAP). The arrows indicate radiolabeled bands coinciding to LHCI immunolabeled bands shown in (IB). The bands in (CAP) are not labeled in the presence of CHX (CHX).

result seems to indicate that the polypeptides are not LHCII, but since the similarity between the green plant and chromophyte LHC proteins is relatively low (Green and Durnford, 1996; Durnford *et al.*, 1999), we probably cannot completely eliminate the possibility that the anti-LHCI is binding LHCII polypeptides. However, just like LHCI, all of the LHCII genes are nuclear encoded in the plants and algae where they have been found (Jansson, 1994, 1999; Wollman *et al.*, 1999), and even if we have identified LHCII, the conclusion is still the same: that an algal LHC gene has been transferred to the DNA of the slug.

The immunoprecipitations provide additional evidence that the LHCI polypeptides are being synthesized on the cytoplasmic ribosomes in the slug. The high amount of radioactivity precipitated by the antibody in the presence of CAP compared to that precipitated in the presence of CHX demonstrates that the proteins recognized by the anti-LHCI are indeed synthesized in the slugs. Since the amount of radioactivity incorporated varied from slug to slug and from experiment to experiment, we had to normalize the immunoprecipitation data as percent of control in order to compare them. However, in a typical experiment, the values for the amount of radioactive material incorporated into the precipitate in the presence of CAP ranged from 5000 to 25,000 cpm, whereas those in the presence of CHX ran from 150 to 400 cpm, which may give a clearer picture of the level of material bound by the antibody.

The results of the pharmacological experiments, the immunoblots, and the immunoprecipitations, taken together, provide substantial evidence that LHCI is the identity of some of the plastid proteins that are synthesized in the presence of CAP. The inhibition of LHCI synthesis by CHX suggests that the algal *Lhca* genes have somehow been transferred to the slug.

To be certain that a gene transfer has occurred, direct evidence of the gene in the genomic DNA of the slug must be found, and we are pursuing this confirmation. However, in addition to the results presented here, other circumstantial evidence for the transfer of the LHCI genes between alga and slug is available in several characteristics of the association. First, although the turnover rate of LHCI in *E. chlorotica* is unknown, the fact that it is synthesized indicates that it is not an unusually robust protein—LHCI replacement is necessary for plastid function to proceed. Second, *Lhca* genes have not been found in the plastid genomes of any organism (Durnford *et al.*, 1999), including other *Vaucheria* species (Linne von Berg and Kowallik, 1992). Of course, if LHCI were present in the plastid genome, it would be synthesized with CHX present, as is the case with the cyt *f* controls; but it is not. Third, the *V. litorea* plastid genome is 119.1 kb (Green *et al.*, 2000), which is similar in size to those of other algae, including *V.*

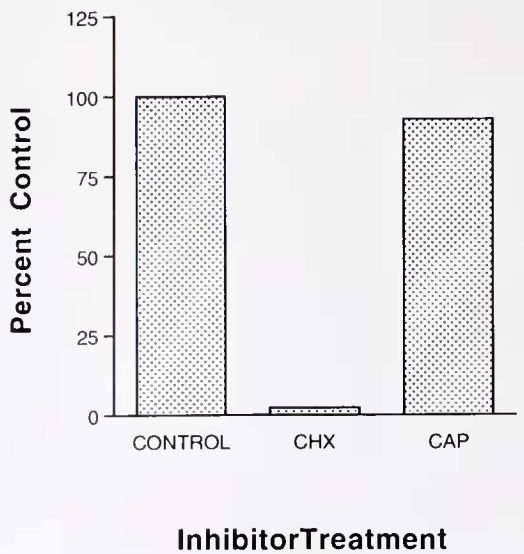

Figure 7. CHX inhibits synthesis of LHCI. In the presence of CHX, anti-LHCI precipitated only 2% of control radioactivity incorporated into LHCI compared to 92% of control in the presence of CAP. Control rates were defined as 100%, and inhibitor rates were calculated as a mean percent of control ($n = 6$).

sessilis and *V. bursata* (Linne von Berg and Kowallik, 1988, 1992), but small relative to those of other plants (Martin and Herrmann, 1998). Even though the plastid genomes of chromophytic algae have a greater coding capacity, relative to their size, than other algae because of fewer introns and inverted repeats (Rieth, 1995), they are too small to carry sufficient genetic information to encode all of the enzymes required for photosynthesis and plastid protein targeting. Fourth, transfer of algal DNA remnants or a nucleomorph-type structure during plastid capture seems unlikely. To date, nucleomorphs have been found only in the Cryptophyta and Chlorarachniophyta (Delwiche, 1999; Zauner *et al.*, 2000) and have not been identified in any chromophyte (Maier *et al.*, 1991; Delwiche, 1999). Although DNA of this type would probably be transcribed on nucleomorph 80S ribosomes (Douglas *et al.*, 1991) and blocked by CHX, neither substantial electron microscopy (Kawaguti and Yamasu, 1965; Graves *et al.*, 1979; Mujer *et al.*, 1996) nor molecular testing (Green *et al.*, 2000) has so far produced evidence for either nucleomorphs or algal nuclear remnants in *E. chlorotica*. Furthermore, if algal DNA remnants were present somewhere in the slug cells, the likelihood is remote of their containing the correct genes and being present in all of the plastid-containing cells in all of the slugs in the populations year after year. Finally, others have suggested that some of the proteins necessary to maintain photosynthesis may be encoded in the mitochondrial genome and are redirected to the chloroplast (Rumpho *et al.*, 2000). Although dual targeting of proteins has been demonstrated in *Arabidopsis* (Chow *et al.*, 1997; Menand *et al.*, 1998), it seems highly unlikely with LHCI. LHCI has never been found associated with mitochondria in any organism; and CAP, which inhibits the mitochondrial ribosomes in addition to those associated with the plastids, would prevent its synthesis anyway.

The horizontal transfer of DNA from the endosymbiont to the nucleus of the host cell provides the basis for the theory of the endosymbiotic origin of eukaryotic organelles. This movement of the symbiont's genes to the host enabled the host to incorporate the organelle's function into its own biochemistry and to faithfully replicate it in subsequent generations. The remnants of eubacterial genes in the mitochondrial and plastid genomes of modern eukaryotes probably resulted from such events (Martin and Herrmann, 1998). Most of the discussions regarding the evolution of plastids focus on the horizontal gene transfer resulting from the primary endosymbiotic event in which a primitive prokaryote engulfed a cyanobacteria (Palmer, 1993; Reith, 1995; Palmer and Delwiche, 1996; Martin *et al.*, 1998; Tengs *et al.*, 2000). Other hypotheses propose a secondary endosymbiosis, probably involving a eukaryote that engulfed a red or green alga (Gibbs, 1981; Palmer and Delwiche, 1996; Martin *et al.*, 1998; Zhang *et al.*, 1999; Delwiche, 1999; Tengs *et al.*, 2000), that produced the plastids of the chromophytic algae and their relatives. In many of these cases, the identity of the initial host, symbiont, or both is unknown. In the case of *E. chlorotica* and *V. litorea*, the origin of LHCI is known; if the gene has been transferred, the transfer occurred between two multicellular eukaryotes and represents a case of tertiary endosymbiosis.

Finally, the mechanism by which such a gene transfer could occur may be found in the viruses that appear in each generation of the slugs at the end of their life cycle. The viruses have several features in common with Retroviridae and seem to be endogenous (Pierce *et al.*, 1999). Retroviruses are capable of transferring genes between organisms; if they are incorporated in the germ cells, they are transferred to the subsequent generations as Mendelian genes (Scharfman *et al.*, 1991). Thus, resolving the relationships between the slugs, alga, plastids, and viruses may have profound implications for both cell and evolutionary biology.

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